13-*cis*-Retinoic Acid Affects Sheath-Shaft Interactions of Equine Hair Follicles *in Vitro*

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A major challenge to the study of hair follicle growth is an appropriate assay system. Because equine mane follicles are large and noncurved, enabling easy dissection; are readily accessible from a single defined source; and possess a long anagen growth phase, we initiated a study of them in culture. As in our previous studies of human and sheep follicles (*Dev Biol* 165:469, 1994), we found in this system that transection level dictates the pattern of follicle growth *in vitro*: follicles transected below the sebaceous gland show a type 1 growth pattern (the shaft grows out with an adherent sheath), while nontransected follicles show a type 2 growth pattern (a naked shaft grows out lacking a sheath). In the present study, we searched for compounds that might influence type 1 or type 2 patterns of growth. We found that 13-*cis*-retinoic acid induced, in a concentration-dependent fashion, a type 1–like pattern of growth under conditions for which a type 2 pattern was expected. All-*trans*-retinoic acid, SR11237 (a synthetic retinoid X receptor-specific ligand), and meta-carboxy-TTNPB (an inactive synthetic retinoid) did not have these properties. We hypothesize that sheath growth/processing is mediated by the follicle at the level of the sebaceous gland, or by the sebaceous gland itself, and that persistence of the follicle sheath about the outgrowing shaft *in vitro* (i) in the physical absence of the sebaceous portion of the follicle, or (ii) in the presence of 13-*cis*-retinoic acid, is due to the reduced expression of a factor that regulates important shaft-sheath interactions. *Key words: sebaceous gland/follicle sheath. J Invest Dermatol 106:356–361, 1996*

The hair follicle offers an instructive model system for the study of secondary induction, heterotypic cell signaling during development, and the controls of complex differentiation pathways. The hair follicle contains seven concentric cylinders of epithelial cells, each layer with its own differentiation pathway leading to distinct cytoskeletal products [1,2]. These epithelial cylinders arise from a deep-lying, basal bulb, which envelops a sphere of mesenchymal cells, the follicular papilla. The papilla directs development of the follicular structure [1], supplying a permissive signal for continued and cyclic growth of the hair [3,4] and apparently influencing the differentiation of the individually distinct epithelial layers. Another epithelial component, the sebaceous gland, develops from the primitive outer root sheath (ORS) of the embryonic follicle [5]. The influence of the sebaceous gland on normal follicular morphogenesis, differentiation, processing, and cycling, though likely important, remains unclear [6].

The first follicular layers to differentiate are those of the inner root sheath (IRS); in fact, Henle's layer of the IRS keratinizes within the upper bulb [7]. The IRS remains tightly packed around the shaft and within the ORS up to the level of the sebaceous gland [8,9]. Rather abruptly, at a level just below the ostium of the sebaceous duct, the keratinized IRS cell layers lose their cohesiveness and slough from the shaft and the ORS to create the pilary canal. *In vitro* follicle growth experiments demonstrate that the release and shedding of the IRS from the shaft and the ORS appear to be an actively regulated process, dependent upon a signal given at the level of the sebaceous gland, rather than a preprogrammed endpoint of differentiation [6]. Transecting an ovine wool or human scalp follicle below the level of the sebaceous gland, which removes the sebaceous gland region of the follicle, results in the adhesion of the sheath to the outgrowing shaft (the type 1 pattern). In contrast, transecting above the level of the sebaceous gland (in addition to not transecting the pilosebaceous unit) leaves the gland and its follicular region intact and results in a naked shaft growing out of the follicle without adherent sheath cells (the type 2 pattern). These experiments suggest that some signal originating within the pilosebaceous unit at the level of the sebaceous gland directs the normal sloughing of the IRS [6]. Earlier, Straile [8] had postulated that sebaceous secretions are actively involved in the normal dissolution of the IRS.

The ability of retinoids to influence epithelial differentiation, especially sebaceous differentiation, *in vitro* and *in vivo*, led us in these experiments to test whether retinoids could alter shaft-sheath relationships *in vitro*. Specifically, we sought to ascertain if any retinoids could shift the type 1 or type 2 patterns of follicular growth.

Retinoids have been reported to have many effects on hair follicles *in vivo*. These effects are both developmentally stage

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Abbreviations: 13cisRA, 13-*cis*-retinoic acid; transRA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; meta-carboxy-TTNPB, m-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl] benzoic acid.

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specific and retinoid structure sensitive. Retinoids applied during early development of the fetal hair follicle cause it to undergo glandular metaplasia forming a mucous-secreting epithelium [10,11]; the competency of embryonic skin to respond this way is lost before birth [10]. A recently described transgenic mouse, containing a dominant-negative retinoic acid receptor gene driven by a K14 promoter, has delayed hair formation [12], which also implicates a role for retinoids in normal hair development.

In these studies, we found that when equine mane follicles are grown in the presence of 13-*cis*-retinoic acid (13cisRA) they manifest a type 1–like growth pattern under conditions for which the type 2 pattern is expected. We describe here the retinoid specificity of this effect and postulate its mechanism of action.

MATERIALS AND METHODS

Animals and Collection of Mane Skin Skin samples were collected from the manes of horses (Standard Bred & Percharon draft, four mares and one gelding, 4–16 y old), which were fed 2 kg of Lampire's custom formulation horse feed daily, one-half a bale of hay daily, and water *ad libitum*. Horses were exposed to the photoperiod and climate of 40°N latitude, pastured 10 h daily, but stabled at night, and cared for by Lampire Biologicals (Pipersville, PA). As follicle pigmentation facilitated dissection, horses chosen as hair follicle donors had darkly pigmented manes. In preliminary experiments, the growth rate of follicles taken from each individual horse was assayed to assure that the donor follicles exhibited a growth rate within an acceptable range (0.7–1.0 mm over 5 d); individual horses that exhibited higher or lower growth rates were not used. Before biopsying, the mane skin of the dorsal medial nuchal ridge was clipped and injected subcutaneously with 1 ml of lidocaine (Xylocaine; Astra Pharmaceutical Products, Westborough, MA). After 5 min, two 6-mm full-thickness biopsies were taken, suspended in Solution A [13] on ice, brought to the dissection laboratory within 1 h of biopsy, and immediately used for follicle preparation.

Hair Follicle Dissection and Growth Measurement Isolation and culture conditions were similar to those described [6,14]. In a dish of cold Solution A, each biopsy was cut orthogonally to the epidermal surface into eight smaller skin pieces. Individual pilosebaceous units were isolated by cutting them free with microscissors. Follicles were removed with their associated sebaceous gland, dermal sheath, and ring of interfollicular epidermis. Follicles visibly damaged during dissection, or removed without either associated sebaceous glands or a continuous ring of interfollicular epidermis, were discarded. Some follicles were transected with microscissors below the level of the sebaceous gland, as performed previously [6].

Follicles were cultured individually in 24-well tissue culture plates (Corning, Corning, NY), containing 1 ml of Philpott's medium [15], which was changed every other day. Culture conditions were 37°C, 5% CO_2/95% air, and 92% humidity. Follicles in plain media were included with each experiment as an internal control. Serial dilutions of retinoids were prepared from stock solutions (10^{-2} M in ethanol) made fresh from compounds kept at −20°C under argon (At the dilutions used, ethanol was found to have no significant effect on follicle growth compared with plain media [data not shown]). All retinoids were handled under yellow safelight (Sylvania Gold fluorescent). Because of potential instability, 13cisRA was used within 4 mo of purchase.

Most media and supplements were obtained from Gibco BRL (Life Technologies, Gaithersburg, MD), except for hydrocortisone (Sigma Chemical Co., St. Louis, MO) and 13cisRA (Acros Organics, Eastman Kodak Co., Rochester, NY). All-*trans*-retinoic acid (TransRA), SR11237, and m-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl] benzoic acid (meta-carboxy-TTNPB) were all provided by Dr. G. Gendimenico of Johnson & Johnson, CPWW (Skillman, NJ). Vitamin A acetate-free Williams' medium was prepared by Gibco.

Most follicles were measured on day 0 and 5 of each experiment. For kinetic experiments, follicles were measured daily for six consecutive days. Measurements were made using a Lietz DM IL inverted microscope (Leica Mikroskopie GmbH, Wetzlar, Germany) and the Java Image Analysis software package (Jandel Scientific, Corte Madera, CA). Two measurements were taken from each follicle: (i) sheath length (from the base of the bulb to the distal end of the sheath) and (ii) shaft extension (from the distal end of the sheath to the end of the shaft). Total length was the sum of these two measurements. Growth calculations and statistical significance were found using Statview 4.01 (Abacus Concepts, Berkeley, CA). Five-day data were analyzed with analysis of variance and Fisher's PLSD post hoc test, while growth curves were analyzed with a repeated measured analysis of variance and a Bonferroni-Dunn post hoc test.

RESULTS

Equine Mane Follicle Growth Except for the fact that the horse cannot be housed in the usual laboratory facility, equine mane skin proved to be an ideal source of anagen follicles: (i) most animals are docile and do not require restraint during the biopsy procedure; (ii) the wound heals rapidly without infection (1 wk); (iii) because these follicles are large and straight, measuring 5.44 ± 0.35 mm (mean ± SEM) in length and 0.40 ± 0.07 mm (mean ± SEM) in diameter, they are easily dissected; and (iv) from each 6-mm punch biopsy one can dissect out from 25 to 30 follicles in 2 h. These follicles have a long anagen growth phase *in vivo* (30-cm terminal hair length) and they show linear growth kinetics for at least 6 d *in vitro* (control media).

While nontransected follicles grow 0.98 ± 0.08 mm (mean ± SEM) over 6 d *in vitro* (**Fig 1*c***, *open circles*), follicles transected below the sebaceous gland grow 0.65 ± 0.10 mm (mean ± SEM) (**Fig 1*f***, *open circles*) over the same time period. After 6–8 d in culture, follicle growth typically degenerates into nonfollicular patterns (i.e., migration of epithelial cells from the follicle to the substrate); for this reason we terminated our growth experiments after 6 d. It is notable that the growth rates of nontransected equine mane, human scalp, and ovine wool follicles are approximately equal over 6 d *in vitro* [6].

Equine mane follicles *in vitro* exhibit the same response to transection as human and ovine follicles [6]. When the follicles are transected below the sebaceous gland they demonstrate a type 1 growth pattern, characterized by shaft outgrowth surrounded by sheath. In contrast, follicles that are not transected demonstrate a type 2 growth pattern, characterized by shaft outgrowth free of sheath. After 6 d in culture, nontransected equine mane follicles show prominent naked shaft outgrowth (0.66 ± 0.07 mm [mean ± SEM]; **Fig 1*b***), a type 2 pattern. In contrast, transecting follicles below the sebaceous gland induces minimal naked shaft outgrowth (0.04 ± 0.01 mm [mean ± SEM]; **Fig 1*e***) and extensive sheath-covered shaft outgrowth **(Fig 1*d*)**, a type 1 pattern.

Effect of 13cisRA on Equine Mane Follicle Growth *in Vitro* Adding 13cisRA to the culture medium significantly promotes sheath outgrowth of nontransected equine mane follicles **(Fig 1*a*)**. However, it does not inhibit naked shaft extension (compare *open triangles* with *open circles* in **Fig 1*b***), and therefore 13cisRA does not strictly convert a type 2 outgrowth to the type 1 outgrowth pattern. Nevertheless, the 13cisRA effect is highly reminiscent of the changes in morphology associated with transection below the sebaceous gland, namely increased sheath outgrowth. Compared with a follicle cultured in control media **(Fig 2*a*)**, 13cisRA causes the infundibulum of the follicle to lengthen prominently over 6 d in culture **(Fig 2*b*)**. Lengthening of the infundibular sheath was observed in 63% of follicles cultured in 10^{-7} M 13cisRA but only 6% of control follicles. Examples of the morphology associated with 13cisRA-induced infundibular lengthening in horse mane follicles are shown in **Fig 3**. The infundibular lengthening can be so dystrophic that a rupture may occur at the level of the infundibulum—for example, as shown in **Fig 3*b***, a day 4 follicle exhibits a ruptured sheath 2 d later **(Fig 3*c*)**, as if the shaft-adherent-sheath stretches to the breaking point due to disparate growth rates.

13cisRA shows a dose-dependent effect on nontransected follicle growth over 5 d in culture **(Fig 4*a*)**. Like all retinoids tested, 13cisRA is toxic to (i.e., blocks) *in vitro* follicle growth at 10^{-5} M, and slightly toxic at 10^{-6} M. Between 10^{-7} and 10^{-10} M it stimulates total linear growth in a dose-dependent fashion, with maximum stimulation at 10^{-9} M. Sheath growth (**Fig 4*a***, *dotted rectangles*) in the presence of 10^{-7}–10^{-11} M 13cisRA is more than doubled over controls. 13cisRA also has an effect on the naked shaft extension of horse follicles *in vitro*; this concentration-dependent response peaks at 10^{-9} M (**Fig 4*a***, *open rectangles*).

It is important to note that 13cisRA does not have any significant effect on the *in vitro* growth of follicles transected below the sebaceous gland (**Figs 1*d–f*, 5**) over a concentration range of 10^{-5}–10^{-11} M **(Fig 5)**. Neither sheath growth **(Fig 1*d*)** nor total

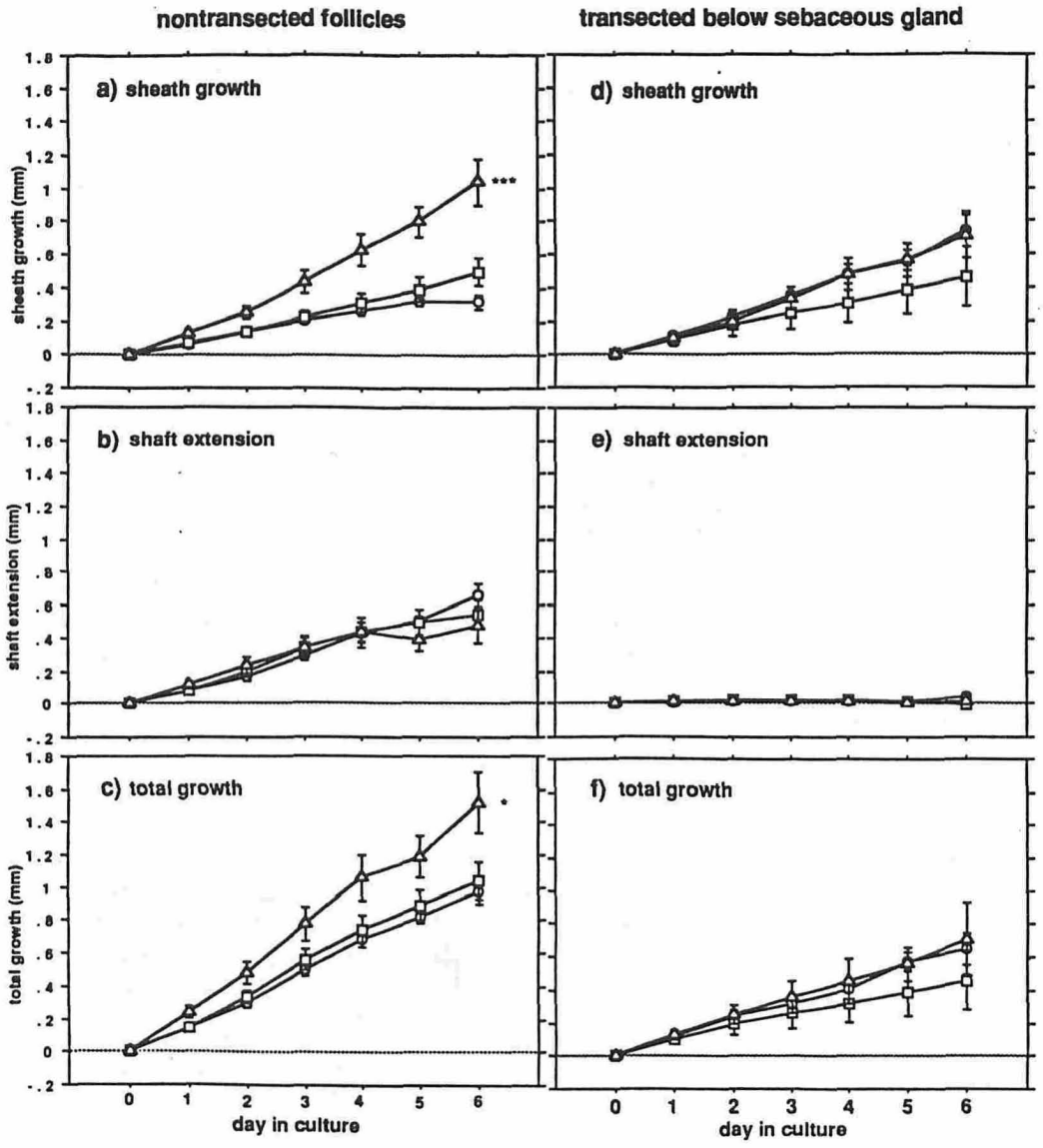

Figure 1. Growth curve of horse mane follicles cultured over 6 d in control media (○), media with 10^{-7} M all-*trans*-retinoic acid (□), and media with 10^{-7} M 13cisRA (△). Follicles are cultured as nontransected pilosebaceous units (*a–c*), or are transected at a level just below the sebaceous gland prior to culture (*d–f*). Graphs show sheath growth (*a,d*), naked shaft extension (*b,e*), or combined total growth (*c,f*) of the follicles. *$p < 0.01$; ***$p < 0.0001$.

growth **(Fig 1*f*)** in the presence of 10^{-7} M 13cisRA are greater than the growth of controls over 6 d.

Morphology of Follicles Cultured in Retinoid-Containing Media The most prominent retinoid effect on follicle morphology is induced by 13cisRA: the infundibular sheath lengthens with the shaft **(Fig 3)**. A second morphological effect of retinoids on follicles in culture is a pinching of the follicular matrix at a level just above the bulb **(Fig 3*a*)**. This effect seems to be due to retinoids at high, but not toxic, doses. Whether this effect is due to an altered differentiation program of the matrix cells has not been ascertained here.

Measuring *in vitro* follicle growth by autoradiography after 2 d in control media *versus* 10^{-7} M 13cisRA did not reveal measurable differences of label uptake in cells of different follicular regions—matrix, sebaceous glands, or ORS (data not shown). This result suggests that the morphological changes caused by retinoids are not due to differential rates of cell division within the progenitor cell populations.

It is notable that none of the compounds studied induced the glandular metaplasia observed in embryonic systems [10].

Effect of Other Retinoids In contrast to 13cisRA, transRA does not significantly increase the *in vitro* sheath growth of nontransected follicles. Moreover, apart from inhibition of hair shaft growth at 10^{-5} and 10^{-6} M, presumably due to retinoid toxicity, transRA has no influence on the rate of follicle growth **(Fig 4*b*)**. Notably, transRA does not stimulate sheath growth between 10^{-7} and 10^{-9} M **(Fig 4*b*, *dotted rectangles*)**.

Since the common medium (Williams' E) used for follicle culture contains 3×10^{-7} M retinyl acetate, retinoid-deficient medium lacking this retinoid was studied. This retinoid-deficient media neither promotes nor inhibits follicle growth after 5 d *in vitro* **(Fig 6)**.

Other retinoids assayed on day 5 of follicle culture show variable influence on sheath growth **(Fig 6)**. The nonactive retinoid, meta-carboxy-TTNPB, has no effect on follicle growth at 10^{-6} and 10^{-7} M concentrations, as observed in other systems [16].

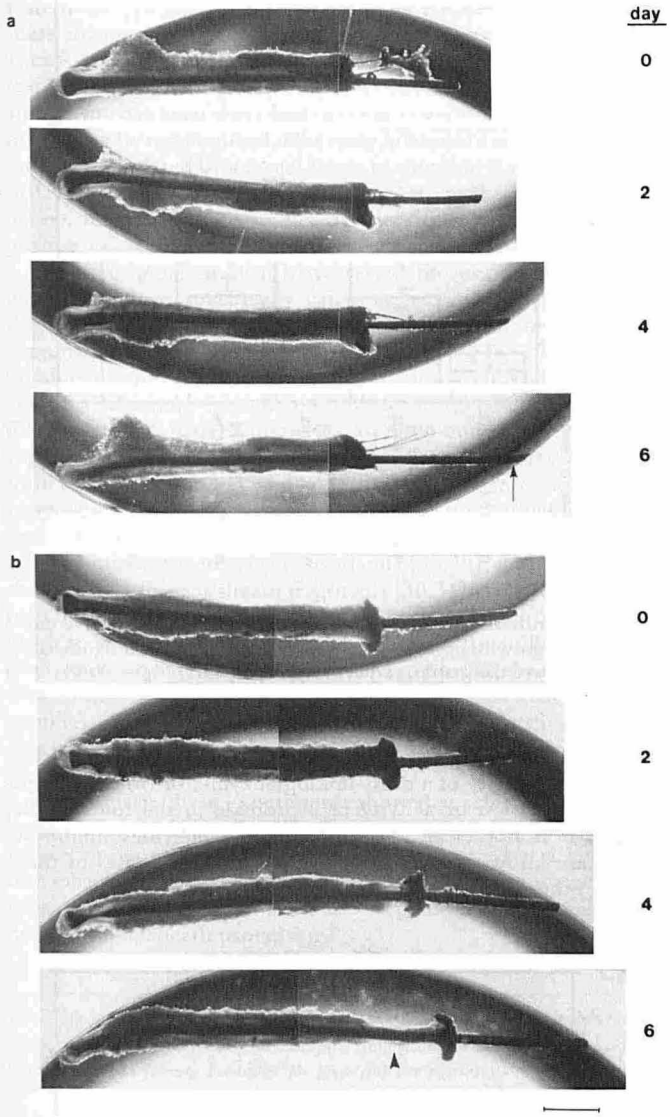

Figure 2. Morphology of horse mane follicles cultured with and without 13cisRA added to the culture medium. The growth of nontransected follicles over time is shown in control medium (*a*), and with 10^{-7} M 13cisRA (*b*). While growth in control medium is characterized by shaft extension (→) and minimal sheath growth, 13cisRA promotes follicular sheath growth in a stretched morphology (▶). *Scale bar*, 1 mm.

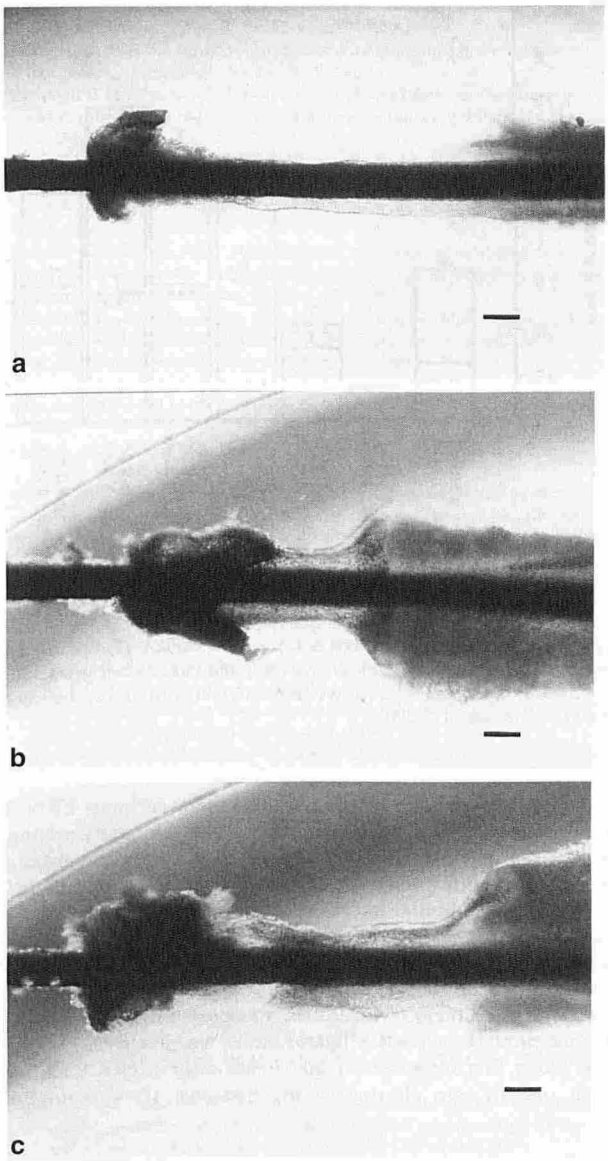

Figure 3. A greatly stretched infundibulum characterizes follicles grown in 13cisRA. *a*) Follicle grown in 10^{-7} M 13cisRA for 6 d shows a pinched syprabulbar matrix. A follicle which is stretched on day 4 (*b*) has ruptured by day 6 (*c*). *Scale bar*, 0.15 mm.

SR11237, a retinoid X receptor–selective agonist [17,18], is inactive over the concentration range tested **(Fig 6)**.

DISCUSSION

In this paper we describe a new model system for studying hair growth *in vitro*. The advantages of using the equine mane follicle to study anagen hair growth include (i) a consistent, abundant, and controllable source of follicles in contrast to a variable human source; (ii) a technically simple dissection procedure in contrast to the dissection of the small, curved ovine wool follicle [6]; and (iii) a good growth response *in vitro* due to its long anagen phase, in contrast to the short growth response of rodent vibrissae [19] (personal observation). Equine mane follicles are similar to human scalp follicles in that both grow to a much greater length than the rest of the pelage, both show comparable growth kinetics and morphology over 6 d in culture [6], and both share a long anagen growth phase *in vivo* which would theoretically maximize the time *in vitro* before the initiation of spontaneous catagen. The primary drawback of an equine *versus* a human experimental system is the limited understanding of horse molecular genetics, though this limitation may be temporary considering the rapid progress being made in the study of chromosomal synteny [20].

Previous work [6] showed that the upper follicle plays an important but mechanistically undefined role in follicle sheath processing. The "normal" type 2 growth pattern of a nontransected follicle in culture, which is reminiscent of *in vivo* growth, becomes a unique type 1 growth pattern, reminiscent only of a few *in vivo* pathologic states, after transection below the sebaceous gland [6]. The type 2 growth pattern appears to be the result of an active process, which entails the limited and specific degradation and sloughing of the sheath at the level of the sebaceous gland liberating a sheathless shaft.

Our previous studies had shown a growth change after physical removal of the sebaceous region; the present experiments show that a similar change in the processing of the sheath can be chemically induced. Nontransected follicles, expected to grow in a type 2 pattern, exhibit much greater sheath growth when exposed to

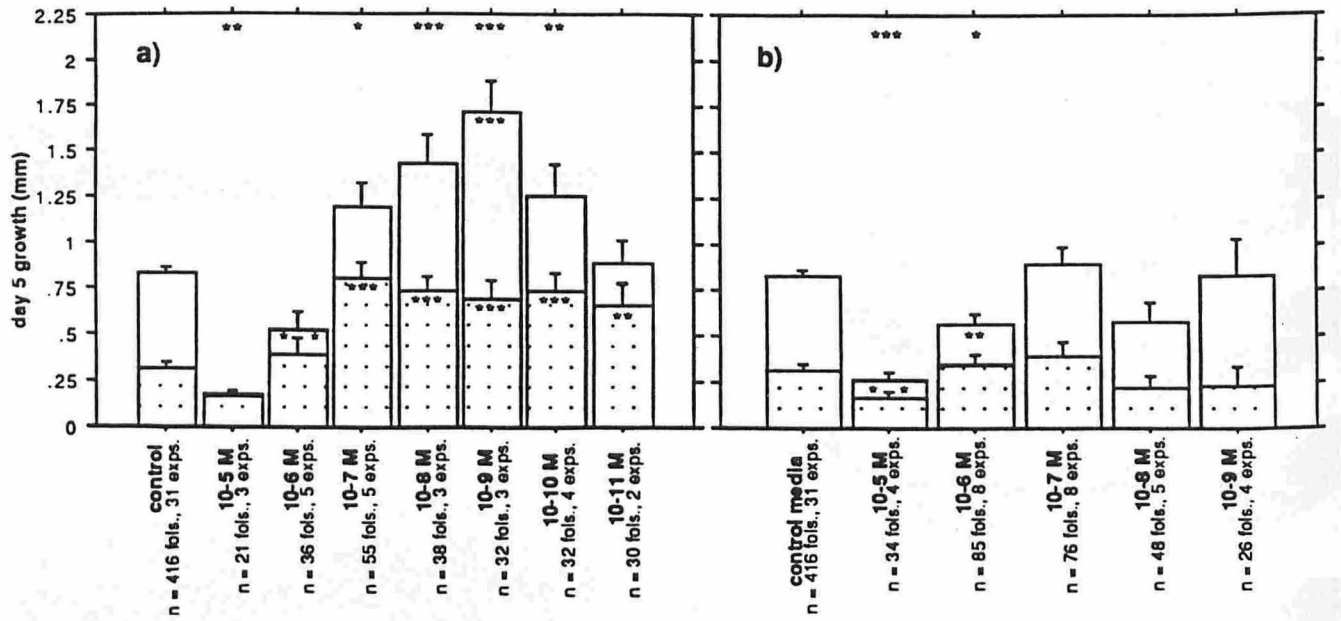

Figure 4. The effect of 13cisRA (*a*) and transRA (*b*) on nontransected horse mane follicle growth. Each bar represents the cumulative day 5 growth, composed of its sheath growth (▨) and naked shaft growth (□). *Lower error bar*, sheath growth; *upper error bar*, total growth. Statistical significances of the sheath, shaft, and total growth measurements (found at the top of ▨, the top of □, and above the graphs, respectively): $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. *Error bar*, 1 SEM.

13cisRA. Since transRA, SR11237, and meta-carboxy-TTNPB do not have this effect, there is clearly chemical selectivity among this group of compounds. The efficacy of 13cisRA in this instance is ascribed to its ability to reduce sebaceous gland secretions [21,22] and to alter infundibular keratinization. The inactivity of SR11237 in this assay suggests that the change in follicle sheath growth observed is mediated by transactivation of retinoic acid receptor–retinoid X receptor heterodimers, rather than by retinoid X receptor homodimers. Moreover, receptor binding itself is not sufficient since the transRA ligand alone was inactive.

It is likely that retention of the sheath after 13cisRA addition is due to one of two alternative mechanisms: (i) upregulation of expression or activity of a cross-linking enzyme, or (ii) downregulation of expression or activity of a protease in the mid follicle. Although it is not clear what the putative enzymes might be, retinoids are known to affect the expression of enzymes of these classes in other systems (e.g., see [23–26]).

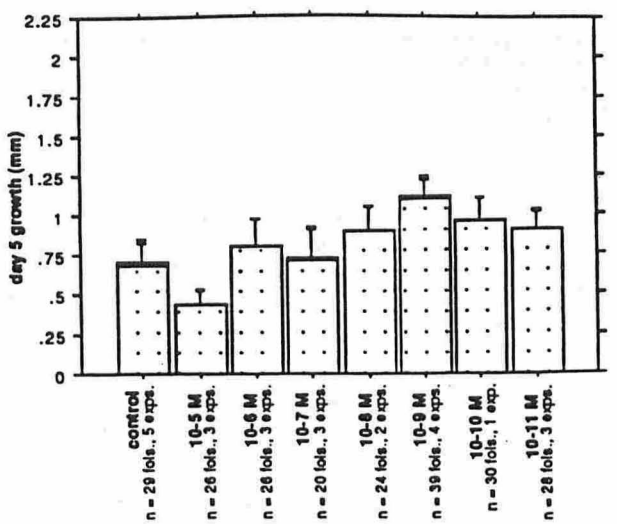

Figure 5. The effect of 13cisRA on the growth of horse mane follicles transected below the level of the sebaceous glands. Each bar represents the cumulative day 5 growth, composed of its sheath growth (▨) and naked shaft extension (□). *Lower error bar*, sheath growth; *upper error bar*, total growth (note: in some cases they overlap). *Error bar*, 1 SEM.

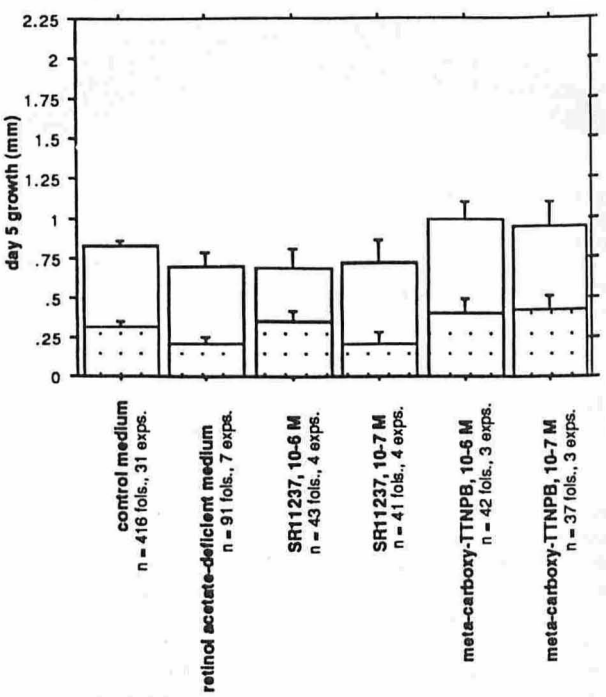

Figure 6. Effect of other retinoids on the sheath (▨), shaft (□), and total (▨ and □ together) growth of nontransected horse mane follicles cultured 5 d *in vitro*. *Lower error bar*, sheath growth; *upper error bar*, total growth. Statistical significance of the sheath and shaft growth measurements compared with control are found at the top of ▨ and the top of □, respectively: $^{*}p < 0.01$; $^{***}p < 0.0001$. *Error bar*, 1 SEM.

The morphological changes we have observed in this *in vitro* system are artificial compared with the native state. The sheath/shaft changes seen *in vitro* have not been described *in vivo* during 13cisRA treatment, though some pathological states suggest this response (e.g., hair casts [27]). Disparity of retinoid effects *in vivo* and *in vitro* has been described in other systems [12]. 13cisRA taken orally may be metabolized differently *in vivo* and *in vitro*, or its action may be modulated by a target tissue in skin that is not present in the isolated follicle system studied here. Despite the exact correspondence, however, these studies do identify a uniquely functioning portion of the follicle.

In our original study [6], we tested the notion that sebaceous gland secretions specifically cause upper sheath degradation and sloughing. That we were unable to muster convincing evidence supporting that concept, by ablating the sebaceous gland itself, may be due to the fact that physical ablation is not as complete as chemical ablation; indeed, the ability of 13cisRA to change the follicle growth pattern might be ascribed entirely to its ability to suppress sebum production. Relevant to this point are (i) that follicles lacking sebaceous glands are rare in nature, an association supporting the importance of this organ to normal hair physiology; and (ii) that the mouse mutant, asebia *(ab/ab)*, which is characterized by miniaturized sebaceous glands [28,29], and emergent hair shafts with adherent sheath fragments [30,31]. Whatever the direct role of the sebaceous gland and its secretions might be, our present work indicates that the sebaceous portion of the follicle (the gland, duct, and associated isthmus) is crucial to normal sheath processing. Our results are consistent with the hypothesis that this region supplies some factor/enzyme that mediates shaft/sheath relationships, effecting the differentiation and sloughing of the sheath characteristic of normal type 2 growth.

In summary, these experiments show that selective retinoids can induce characteristic sheath growth patterns that have been observed previously in cultured follicles transected at a level below the sebaceous gland. The studies reinforce the importance of the sebaceous gland and/or the sebaceous gland region of the follicle in normal shaft/sheath processing.

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